A Soluble Cytochrome containing c-Type and a_2 -Type Haem Groups from *Micrococcus denitrificans*

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There are relatively few reports of cytochrome pigments, other than the c-type, that are readily extracted from the membrane structures of the cell, or that occur in the soluble fraction. During the isolation and purification of soluble cytochrome c from broken-cell suspensions of anaerobically nitrate-grown *Micrococcus denitrificans*, large quantities of a brown protein and smaller quantities of a blue protein were found in the crude cytochrome c fraction (Smith, Newton & Scholes, 1966). The present communication reports the isolation and partial purification of the brown protein and the blue protein and studies on the nature of the prosthetic groups of the brown protein and the possible role of this protein in electron transport.

Preparations. *M. denitrificans* A.T.C.C. 13543 was grown anaerobically in the presence of nitrate (Vernon, 1956). The washed cells were treated with lysozyme, the protoplasts lysed by osmotic 'shock' and the membrane fragments containing the particle-bound cytochromes were separated from the soluble proteins by centrifugation (Smith *et al.* 1966). The soluble fraction was subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation and DEAE-cellulose column chromatography by modifications of procedures described for *Rhodospirillum rubrum* cytochrome c and cytochrome c-551 from a halotolerant *Micrococcus* (Vernon & Kamen, 1954; Hori, 1961).

The brown protein was precipitated between 75 and 90% saturation with $(\text{NH}_4)_2\text{SO}_4$, and purified by repeated chromatography on DEAE-cellulose with ammonium acetate buffers at pH 6.0; 0.5M-ammonium acetate buffer was required for elution.

The blue protein was precipitated together with cytochrome c by the addition of 0.1 vol. of cold 0.5N-trichloroacetic acid to the 90%-saturated- $(\text{NH}_4)_2\text{SO}_4$ supernatant. The precipitate was dissolved in 0.05M-potassium phosphate buffer, pH 7.0, dialysed against 0.01M-ammonium acetate buffer, pH 6.0, and chromatographed on DEAE-cellulose pre-equilibrated in the 0.01M-ammonium acetate buffer. The blue protein was eluted with 0.1–0.2M-ammonium acetate buffer, pH 6.0, just ahead of

the cytochrome c, which required 0.2–0.3M-ammonium acetate buffer.

Blue protein. The absorption spectrum of the oxidized (untreated) form of the blue protein showed a broad band in the visible region at 595 m μ and a protein band at 277 m μ . There was also some less intense absorption between 400 and 450 m μ , probably caused by impurities since the ratio of this absorption to that at 595 m μ was variable among different preparations. On reduction with dithionite the blue colour and all absorption in the visible region disappeared. The absorption spectra of the compound resemble those of the blue copper protein isolated and crystallized from *Pseudomonas aeruginosa* by Horio (1958), although the single broad band of the *Pseudomonas* pigment is at 630 m μ . Insufficient material was available for further purification or enzymic studies.

Brown protein. The reduced-minus-oxidized difference spectrum of the brown protein is complex and unlike that of a single haem compound, but fewer absorption bands are seen in the CO-reduced-minus-reduced difference spectrum (Fig. 1). Thus it would appear that the brown protein has at least two chromophores, of which only one is CO-reactive.

Fig. 1. Reduced-minus-oxidized (-----) and CO-reduced-minus-reduced (—) difference spectra of brown protein from *M. denitrificans*. Spectra were recorded in 0.1M-phosphate buffer, pH 7.0, and the solutions were diluted fivefold for the reduced-minus-oxidized spectrum in the 400–450 m μ region. $\text{Na}_2\text{S}_2\text{O}_4$ was used for reduction, and CO gas was bubbled through the reduced sample for approx. 2 min. Spectra were recorded on a Cary model 14 R spectrophotometer.

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Table 1. *Comparison of absorption spectra of brown proteins and their derivatives from Micrococcus denitrificans and Pseudomonas aeruginosa*

All spectra were recorded with a Cary model 14R spectrophotometer. $\text{Na}_2\text{S}_2\text{O}_4$ was used for reduction, and unless otherwise stated the pH was 7.0 and the experimental conditions were those described by Yamanaka & Okunuki (1963*a,b*). The data for the *Pseudomonas* pigment were obtained from the same source. Numbers in parentheses refer to minor bands or shoulders in a spectrum.

Spectrum	Maxima and minima (m μ)	
	<i>Micrococcus</i> brown protein	<i>Pseudomonas</i> brown protein
Oxidized pigment, pH 7.0	407, 525, 638, 700	412, 525, 635
Reduced pigment, pH 7.0	416, (460), 521, 547, 554, (615), 653	418, (460), 523, 549, 554, 629, 652
Reduced pigment, pH 5.6	416, (460), 521, 547, 554, 615, (650)	418, (460), 523, 549, 554, 625
Reduced pigment, pH 7.6	416, (460), 521, 547, 554, (615), 653	418, (460), 523, 549, 554, (615), 655
Reduced pigment in 0.25 N-NaOH	416, (460), 521, 559-552, 625	
Reduced minus oxidized		
Maxima	420, 463, 520, 547, 553, 615, 655	418, 460, 520, 549, 554, 615, 670
Minima	403, 440, 635, 705	445, 635
CO-reduced minus reduced		
Maxima	414, (428), 540-570, 680	415, (430), 540-570, 675
Minima	424, 460, 600-665	420, 460, 600-665
CN-reduced minus reduced		
Maxima	447, 480, 627	445, 480, 627
Minima	461, 664	460, 665
Reduced plus nitrite	416, 521, 547, 554, 665	418, 523, 549, 554, 665
Pyridine haemochromogen, alkaline	413, (460), 520, 549, 617	
Cyanide haemochromogen	417, (440), (470), 521, 547, 553, 625	418, (443), (472), 523, 549, 554, 627
Residue after acid-acetone treatment		
Reduced minus oxidized	416, 521, 547, 554	418, 523, 549, 554
Pyridine haemochromogen, alkaline	414, 519, 549	549
Supernatant after acid-acetone treatment		
Ferric haemin, acid	432, (533), (560), (605)	432, (529), (569), (606)
Ferric haem, alkaline	406, (480), 681	405, (480), 684
Ferrous haem, alkaline	455, 620	453, 626
Pyridine haemochromogen, alkaline	617	620
Cyanide haemochromogen	632	632

These spectra are remarkably similar to, but not identical with, those of a soluble protein isolated from nitrate-grown *P. aeruginosa* and studied by Yamanaka and his co-workers (Yamanaka, 1963). Further studies showed that the reduced spectrum in the 600-650 m μ region was pH-dependent, and that the nitrite and cyanide derivatives were also very similar to those of the *Pseudomonas* pigment (Table 1).

Horio, Higashi, Yamanaka, Matsubara & Okunuki (1961) concluded that the complex absorption spectra of the *Pseudomonas* brown protein resulted from the presence of two prosthetic groups, haem *d*(a_2) and haem *c*; Yamanaka & Okunuki (1963*b*) split these two prosthetic groups from the protein and partially characterized them. Similar experiments showed the presence of *c*-type and a_2 -type haem groups in the *Micrococcus* brown protein. A small sample of the concentrated protein was treated with 0.12 N-HCl in acetone at -12°. The flocculent precipitate was removed by centrifugation and suspended in 0.2 M- K_2HPO_4 and then the

pH was adjusted to 9.0. The reduced-minus-oxidized difference spectrum of this solution showed a Soret band at 416, β -band at 521 and two α -peaks at 547 and 554 m μ . However, in the presence of alkaline pyridine and dithionite, the two α -peaks were replaced by a single band at 549 m μ , characteristic of *c*-type haemochromogens (Table 1). The spectrum of the acid-acetone supernatant was almost identical with that of the green haemin prepared from the *Pseudomonas* pigment (Yamanaka & Okunuki, 1963*b*); further, the alkaline ferric and ferrous forms and the cyanide and pyridine haemochromogen derivatives also resembled closely the corresponding derivatives of the *Pseudomonas* pigment (Table 1).

Oxidase activity of the brown protein. The *Pseudomonas* brown protein has both cytochrome *c* oxidase and nitrite reductase activity with *Pseudomonas* cytochrome *c*-551 as electron donor (Yamanaka, 1963), although the turnover rate as an oxidase of 10 sec.⁻¹ at 27° with 14 μM -cytochrome *c* is rather low. The purified brown protein from *M.*

denitrificans showed cytochrome *c* oxidase activity with both mammalian and *Micrococcus* cytochrome *c*. When it was assayed by the spectrophotometric method of Smith & Conrad (1956) the turnover rate was very low: 0.6 sec^{-1} at 25° with *Micrococcus* cytochrome *c* ($16 \mu\text{M}$). The oxidation of soluble cytochrome *c* by the brown protein was a first-order reaction with respect to ferrocytochrome *c*, like the reaction of the membrane-bound oxidase from *M. denitrificans* (Smith *et al.* 1966) and also the mammalian oxidase (Smith & Conrad, 1961). As with the membrane-bound oxidases, the rate constant with the soluble pigment decreased with increasing concentration of cytochrome *c*. With the soluble pigment the decrease in rate constant was such that the velocity remained constant between $4 \mu\text{M}$ - and $60 \mu\text{M}$ -cytochrome *c*. The reaction was also extremely sensitive to ionic strength, but showed no pH optimum in buffers of constant ionic strength. The oxidase activity of the purified brown protein was inhibited 100% by mM-KCN and 50% by 10mM-azide. The azide inhibition was gradually reversed with time.

Discussion. The above evidence suggests that the brown protein isolated from anaerobically nitrate-grown *M. denitrificans* is a cytochrome pigment containing both haem *c* and a *d(a₂)*-like haem. In this respect it closely resembles the brown protein studied by Yamanaka and co-workers in nitrate-grown *P. aeruginosa*. Until further chemical studies are carried out, it is not correct to assume that the green haem from either organism is identical with the haem *d(a₂)* originally isolated from *Aerobacter aerogenes* and described by Barrett (1956). Yamanaka (1963) has discussed a role of an oxidase and a nitrite reductase for the *Pseudomonas* protein. The latter seems more important, as the presence of this protein and of nitrite reductase activity is dependent on the presence of nitrate in the medium and not on aerobic or anaerobic growth. The

possibility that the *Micrococcus* brown protein might function as a nitrite reductase has not yet been fully investigated, although the fact that a nitrite derivative was demonstrated spectrophotometrically (Table 1) supports this idea. A significant function as a terminal oxidase seems unlikely, as the membrane fragments of both aerobic and anaerobic preparations contain an extremely efficient cytochrome *a + a₃* oxidase system (Smith *et al.* 1966) and there is no evidence for *a₂*-type cytochromes in anaerobic-minus-aerobic difference spectra of cells grown with nitrate either aerobically or anaerobically (P. B. Scholes & L. Smith, unpublished work).

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